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13. ABSTRACT ***(Maximum 200 Words)***

One of the central anti-apoptotic pathways induced by diverse cellular stresses is mediated by NF-κB transcription factors. The overall aim of this project is to understand the role of up-stream IkappaB kinases and NF-κB "survival signaling" pathway in tumorigenesis in prostate. During the current funding period we focused on NF-κB activity in normal prostate epithelial cells and in a panel of androgen-dependent and androgen-independent prostate carcinoma (PC) cell lines. Our results indicated that NF-κB was constitutively activated in human androgen-independent PC cell lines. We found that the level of IκBα phosphorylation and the rate of IκBα degradation were increased in PC cells with high constitutive NF-κB activity due to the constitutive activation of IKKs. We have developed a comprehensive picture of NF-κB induction in different prostate cell lines by diverse NF-κB activators. It appeared that in spite of the high constitutive level of NF-κB activity, NF-κB in general is readily inducible in PC cells. Results of our experiments also showed that selenium, which is an effective preventive agent for PC, inhibited IKK and NF-κB activity and induced apoptosis in PC cells. Next year we will study the role of specific IKKs in constitutive activation of NF-κB in PC cell lines and PC tumors.

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Table of Contents

Introduction

The overall aim of this project is to understand the role of up-stream IkappaB kinases (IKK) and NF-κB "survival signaling" pathway in tumorigenesis in prostate. During the current funding period we focused on evaluation of NF-κB/IκB protein expression and NF-kB activity in normal prostate epithelial cells and in a panel of androgen-dependent and androgen-independent prostate carcinoma (PC) cell lines. We also studied sensitivity of PC cells to NF-kB induction and inhibition, and the consequences of NF-kB inhibition in PC cells.Our results indicated that NF-κB was constitutively activated in human androgen-independent PC cell lines due to the constitutive activation of IKKs. Results of our experiments also showed that selenium, which is an effective preventive agent for PC in humans, inhibited IKK and NF-κB activity and induced apoptosis in PC cells. Our studies have resulted in publication of one peer-reviewed publication. One more manuscript has been submitted. The following describes progress made in this year.

Body

As was proposed we focused during first year on the tasks 1-5 pertinent to the first part of the Specific Aim 1 of proposal to develop a comprehensive picture of expression/function of IKKs in PC cell lines and normal prostate cells in vitro. We have also started to build a collection of frozen and formalin fixed samples of PC tumors as proposed in task 3 to extend our findings in vitro for prostate tumors. In addition, we have also begun to perform stabile and transient transfections of PC cell lines with IKKα, IKKβ, IKKγ and IKKε d.n. constructs as was proposed in tasks 6 and 8.

We have investigated the expression and function of NF-κB, IκB inhibitors and IKKs in normal prostate epithelial cells and prostate carcinoma (PC) cell lines LNCaP, MDA PCa 2b, DU145, PC3, and JCA1. We found that NF-κB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, and JCA1. NF-κB activity was evaluated using electrophoretic mobility shift assay (EMSA), transient transfections with κB.Luciferase reporter, and Northern blot analysis of endogenous κB-responsive genes such as IκBa and IL-6. In spite of strong difference in constitutive κB binding, Western blot analysis did not reveal any significant variance in the expression of p50, p65, IκBs, IKKα, and IKKβ between primary prostate cells, androgen-dependent and androgen-independent PC cells. The direct sequencing of IκBα cDNA has not predicted any amino-acid substitutions in IκBα protein in those cell lines with constitutive NF-κB activation.

However, we found that in androgen-independent PC cells IκBα was heavily phosphorylated and displayed a faster turnover. By an in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent DU145, PC3 and JCA1 PC cell lines. To further explore the role of IKKs in constitutive NF-κB activation in malignant prostate cells we studied the effect of kinase-inactive mutants of either IKKα (IKKαK44M) or IKKβ (IKKβK44M) on the constitutive NF-κB transcription activity in normal and malignant PC cells in comparison to the effect of IκBα super repressor. We found that both IKK mutants inhibited constitutive luciferase activity in normal and malignant PC3 prostate cells in a similar way, with IKKβ mutant being more potent inhibitor for constitutively active NF-κB.

Blockage of NF-κB activity in PC cells by d.n.IκBα resulted in increased constitutive and TNF-α-induced apoptosis. We also studied the effect of Selenium, which is an effective preventive agent for PC in humans, on NF-κB activity, IKK and apoptosis in PC cells. Using sodium selenite and a novel synthetic compound methylseleninic acid (MSeA) that served as a precursor of the putative active monomethyl metabolite methylselenol, we found that both Se forms inhibited NF-κB DNA binding induced by TNF-α and LPS in DU145 and JCA1 cells. We also showed that selenite and MSeA inhibited IKK activation, IκB-α phosphorylation and degradation induced by TNF-α and LPS in prostate cells. The extent and persistence of NF-κB inhibition appeared to correlate with the Se effect on growth and survival of prostate cells.

Key Research Accomplishments

- ❖ NF-kB is constitutively activated in human androgen-independent PC cell lines.
- ❖ There are no alterations in the expression of NF-kB and IkB proteins and no mutations in IkBa gene in PC cell lines with constitutively active NF-kB.
- ❖ The levels of IkBa phosphorylation and degradation are increased in androgen-independent malignant PC cells due to constitutive activation of IKK.
- ❖ In spite of the high level of constitutive activity, NF-kB is readily inducible in PC cells.
- ❖ Different inhibitors of NF-kB inhibited PC cell growth and induced apoptosis in PC cells.

Reportable outcomes

Manuscripts

1. Gasparian, A.V., Yao, Y., Kowalczyk, D., Lyakh, L. A., Karseladze, A., Slaga, T.J.,and Budunova, I.V. Mechanisms of constitutive NF-κB activation in prostate carcinoma cells. Journal of Cell Science, 115: 141-151, 2002.
2. Gasparian, A.V., Yao, Y.J, Lu, J., Slaga T.J, and Budunova, I.V. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cells. Submitted.

Abstracts presented at national meetings

1. Gasparian, A. V., Yao, Y. J., Lu, J., Slaga, T. J., and Budunova I. V. Chemopreventive properties of Selenium compounds are associated with inhibition of IκB kinase and transcriptional factor NF-κB. Proceedings of AACR. 21*supplement:* 78, 2001.
2. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V. Mechanism of constitutive NF-κB activation in prostate carcinoma cells. Proceedings of AACR. *21:* 702, 2001.
3. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V. NF-κB is constitutively activated in prostate carcinoma cells. Abstracts of International Symposium: NF-kB regulation, Gene expression and Disease. July 4-8, 2001, Gent, Belgium, p.17.
4. Gasparian A.V., Yao Y.J., Lü J., Slaga T.J. and Budunova I.V. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Keystone Symposium: NF-kB: bench to bedside. February 25-March 3, 2002, Keystone, Colorado, p. 60.

Seminar presentations by PI

1. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Chemicon International Inc, Temecula, CA. August, 2001.

2. Mechanism of constitutive NF-kB activation in prostate carcinoma cells. Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH. October 2001

3. Constitutively active NF-κB transcription factor in prostate carcinoma cells as a possible target for intervention. Graduate Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO. November 2001.

4. NF-κB transcription factor and IKK kinases in prostate carcinoma cells as a possible target for intervention. Grand rounds, Department of Pathology, School of Medicine at the University of Colorado. Denver, CO. November 2001.

5. NF-κB transcription factor and IKK kinases in prostate carcinoma cells as a possible target for intervention. UT M.D. Anderson Cancer Research Center, Department of Carcinogenesis, Smithville, TX. January 2002.

6. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Basic Science Conference, Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, CO. February 2002.

Conclusions

Our data suggest that increased IKK activation leads to the constitutive activation of NF-κB "survival signaling" pathway in androgen-independent PC cells. This may be important for the support of their androgen-independent status and growth advantage.

Our results also suggest that Se may target the NF-kB activation pathway to exert, at least in part, its cancer chemopreventive effect in prostate.

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3. Barkett, M. and Gilmore, T. D. Control of apoptosis by Rel/NF-kappaB transcription factors, Oncogene. *18:* 6910-24, 1999.

Appendices

1. Gasparian, A.V., Yao, Y., Kowalczyk, D., Lyakh, L. A., Karseladze, A., Slaga, T.J.,and Budunova, I.V. Mechanisms of constitutive NF-κB activation in prostate carcinoma cells. Journal of Cell Science, 115: 141-151, 2002.
2. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V. NF-κB is constitutively activated in prostate carcinoma cells. Abstracts of International Symposium: NF-kB regulation, Gene expression and Disease. July 4-8, 2001, Gent, Belgium, p.17.
3. Gasparian A.V., Yao Y.J., Lü J., Slaga T.J. and Budunova I.V. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Keystone Symposium: NF-kB: bench to bedside. February 25-March 3, 2002, Keystone, Colorado, p. 60.

The role of IKK in constitutive activation of NF-κB transcription factor in prostate carcinoma cells

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Summary

Rel/NF-κB transcription factors are implicated in the control of cell proliferation, apoptosis and transformation. The key to NF-κB regulation is the inhibitory IκB proteins. During response to diverse stimuli, IκBs are rapidly phosphorylated by IκB kinases (IKKs), ubiquitinated and undergo degradation. We have investigated the expression and function of NF-κB, IκB inhibitors and IKKs in normal prostate epithelial cells and prostate carcinoma (PC) cell lines LNCaP, MDA PCa 2b, DU145, PC3, and JCA1. We found that NF-κB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, JCA1 as well as androgen-independent CL2 cells derived from LNCaP. In spite of a strong difference in constitutive κB binding, Western blot analysis did not reveal any significant variance in the expression of p50, p65, IκBs, IKKα, and IKKβ between primary prostate cells, androgen-dependent and androgen-independent PC cells. However, we found that in androgen-independent PC cells IκBα was heavily phosphorylated and displayed a faster turnover. Using an in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent PC cell lines. Blockage of NF-κB activity in PC cells by dominant-negative IκBα resulted in increased constitutive and TNF-α-induced apoptosis. Our data suggest that increased IKK activation leads to the constitutive activation of NF-κB 'survival signaling' pathway in androgen-independent PC cells. This may be important for the support of their androgen-independent status and growth advantage.

Key words: NF-κB, IκBα phosphorylation, IKK, Prostate cancer

Introduction

The signaling pathways that regulate cell proliferation, survival and transformation are of prime interest in cancer biology. Recently, the Rel/NF-κB transcription factors, the known regulators of immune and inflammatory responses, have been found to be critically important for control of cell proliferation, apoptosis and tumor development (Rayet and Gelinas, 1999). The Rel/NF-κB transcription factors are homo- and heterodimers consisting of proteins from the Rel/NF-κB family. In mammals the Rel/NF-κB family includes five proteins: NF-κB1 (p50/105), NF-κB2 (p52/100), RelA (p65), RelB and c-Rel. In unstimulated cells NF-κB is sequestered in the cytoplasm by inhibitory molecules, IκBα, IκBβ, IκBε, as well as precursors of NF-κB1 and NF-κB2, proteins p105 and p100. Most agents that activate NF-κB employ a common pathway based on the phosphorylation of the two N-terminal serine's in IκBs, with subsequent ubiquitination and degradation of these proteins by the 26S proteasome (Whiteside and Israel, 1997; Heissmeyer et al., 1999). The released NF-κB factors then translocate to the nucleus and activate transcription of κB-responsive genes. Signal-induced phosphorylation of IκBs is executed by a 900 kDa complex called 'signalosome' containing two inducible IκB kinases IKKα and IKKβ, as well as several structural proteins (Zandi et al., 1997; Yamaoka et al., 1998)

Several lines of evidence suggest that aberrant NF-κB regulation is associated with oncogenesis in mammalian systems. Amplification, overexpression or rearrangement of all genes coding for Rel/NF-κB factors with exception of RelB have been found in leukemias and lymphomas (Rayet and Gelinas, 1999). Constitutive activation of NF-κB is a common characteristic of many cell lines from hematopoietic and solid tumors (Rayet and Gelinas, 1999; Baldwin, 1996; Wang et al., 1999; Dejardin et al., 1999; Bours et al., 1994; Nakshatri et al., 1997; Sovak et al., 1997; Visconti et al., 1997, Palayoor et al., 1999; Duffey et al., 1999; Barkett and Gilmore 1999). The blockage of NF-κB activity in carcinoma cell lines by different approaches dramatically reduced their ability to form colonies in agar and reduced their growth in vitro and in vivo (Visconti et al., 1997; Duffey et al., 1999). It is important that NF-κB also plays a key role in cell protection against diverse apoptotic stimuli including chemotherapeutic drugs and γ-irradiation through activation of the anti-apoptotic gene program in cells (Barkett and Gilmore, 1999).

In spite of the growing evidence of the important role of NF-κB in tumorigenesis and resistance to chemotherapy, only a few attempts have been made to analyze the mechanisms of constitutive activation of NF-κB in transformed cells. It was found that mechanisms involved in NF-κB activation in tumor cell lines could be different, and include increased expression of NF-κB proteins, especially p50 and p52, mutations and deletions in IκBα gene and increased IκBα turnover

(Devalaraja et al., 1999; Krappmann et al., 1999; Budunova et al., 1999; Rayet and Gelinas, 1999).

The aim of this study was to develop a comprehensive and detailed picture of changes in basal NF-κB activity in a panel of prostate cells including primary prostate epithelial cells and six prostate carcinoma (PC) cell lines, and to elucidate the molecular mechanisms that could account for the NF-κB activation in PC cells, including the level of expression of Rel/NF-κB proteins, mutations in the IκBα gene, and IκBα turnover. Our results indicate that NF-κB is constitutively activated in human androgen-independent PC cells. We did not reveal any significant differences in the expression of various NF-κB and IκB proteins or IκBα mutations in any of the examined cell lines. Instead, in androgen-independent PC cells IκBα was heavily phosphorylated and displayed a shorter half-life. Our results indicate that aberrant IKK activation in androgen-independent PC cells leads to the constitutive activation of NF-κB 'survival signaling' pathway, possibly contributing to their growth advantage.

Materials and Methods

Cell cultures and treatments

LNCaP, MDA PCa 2b, DU145 and PC3 cells were purchased from American Type Culture Collection (Rockville, MD), JCA1 cells (Muraki et al., 1990) were received from O. Rokhlin (University of Iowa, Iowa City, IA). The androgen-independent CL2 cells derived from LNCaP cells via an in vitro androgen deprivation, were received from A. Belldegrun (Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA). Primary prostate epithelial cells were purchased from Clonetics Corporation (Walkersville, MD). LNCaP, CL2, DU145, PC3 and JCA1 were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 1 mM sodium pyruvate (Sigma Chemical Co., St Louis, MO), 0.1 mM β-mercaptoethanol (Sigma) and antibiotics. Primary prostate epithelial cells and MDA PCa 2b were cultured in the media and under conditions recommended by ATCC and Clonetics Corporation accordingly. Cells at 80% confluency were treated with 10 μg/ml cycloheximide (CHX) (Biomol Research Laboratories, Inc., Plymouth, PA), 7.5 μg/ml MG132 (Biomol Research Laboratories Inc.) or 3.2 μg/ml 15-deoxy-Δ^{12-14}-prostaglandin J2 (Cayman Chemical Company, Ann Arbor, MI).

Preparation of cellular extracts and electrophoretic mobility shift and supershift assays (EMSA and EMSSA)

Nuclear and cytosolic proteins were isolated as described previously (Lyakh et al., 2000). The binding reaction for EMSA contained 10 mM Hepes (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, [γ-^{32}P]-labeled (2-3×10^5 cpm) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI), [γ-^{32}P]-labeled (2-3×10^5 cpm) double-stranded oligonucleotide representing Sp1-consensus binding site (Santa Cruz Biotechnology, Santa Cruz, CA), and 5-10 μg of the nuclear extract. DNA-binding reaction was performed at room temperature for 30-45 minutes in a final volume of 20 μl. For EMSSA antibodies against p65 (sc-109X), p50 (sc-114X), p52 (sc-298X), c-Rel (sc-71X) or RelB (sc-226X), were added 30 minutes after the beginning of reaction, and incubation was continued for an additional 30-45 minutes. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-protein complexes were analyzed on 6% DNA retardation gels (Novex, Carlsbad, CA). Dried gels were subjected to radiography.

Western blot analysis

Proteins were resolved by electrophoresis on 10-12.5% SDS-PAAGs and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA). Polyclonal anti-p50 (# 06-886), anti-p52 (# 06-413) anti-c-Rel (# 06-421) antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-p65 (sc-372), anti-RelB (sc-226) anti-IκBα (sc-371), anti-IκBβ (sc-946), anti-IκBε (sc-7156) or anti-IKKα/β (sc-7607) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser32 IκBα Ab was from Cell Signaling Technology Inc. (Beverly, MA). Anti-PARP Ab was from PharMingen (San Diego, CA). Membranes were blocked with 5% nonfat milk in TBST buffer and incubated with primary antibodies for 1.5 hours at room temperature. Anti-Phospho-Ser32 IκBα Ab required 6 hours incubation at 34°C. Peroxidase-conjugated anti-rabbit IgG (Sigma) was used as a secondary antibody. ECL Western blotting detection kit (Amersham Pharmacia Biotech, Sweden) was used for protein detection. The membranes were also stained with Ponceau Red to verify that equal amounts of proteins were loaded and transferred.

Pulse-chase analysis of IκBα degradation

Metabolic labeling of cells was performed as described previously (Krappmann et al., 1999). Protein extracts were prepared at the indicated time points. Cells were washed twice with cold PBS and lysed in TNT buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100) with protease inhibitors as described previously (Lyakh et al., 2000). Lysates were incubated on ice for 15 minutes and then centrifuged for 5 minutes at 13,000 ***g***. Supernatant was used for immunoprecipitation. Immunoprecipitation of 400 μg of the total protein in 3 ml of TNT buffer was performed using IκBαN (#1309) antiserum (a generous gift from N. Rice, NCI, Frederick, MD). Two hours later 20 μl of protein A-sepharose 4B (Sigma Chemical Co.) in TNT buffer were added to each sample and incubated with gentle rotation overnight. Then sepharose beads were washed 5 times with ice-cold TNT buffer and boiled for 5 minutes in SDS-loading buffer. The supernatant was resolved by SDS-PAAG followed by transfer to Immobilon-P membrane (Millipore Corporation).

In vitro IKK activity assay

Unstimulated prostate cells and LNCaP cells treated with TNF-α (7.5 ng/ml) were lysed in TNT buffer with protease inhibitors. Immunoprecipitation of 450 μg of total protein was performed with 1 μl of rabbit IKKα (#1997) and IKKβ (#4137) antisera (a kind gift of N. R. Rice, NCI, Frederick, MD), as described for pulse-chaise assay. Immunoprecipitate was washed three times in TNT buffer with protease inhibitors and twice with kinase buffer without protease inhibitors. Kinase reaction was performed in kinase buffer (20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 2 mM $MnCl_2$), containing 2 μCi of [γ-^{32}P]ATP and IκBα peptide (1-54) that has only Ser32 and Ser36 sites of phosphorylation (Boston Biologicals Inc., Boston, MA) as a substrate for 30 minutes at 30°C. Then 2× Tricine/SDS sample buffer (Novex, Carlsbad, CA) was added to each reaction, samples were boiled and subjected to PAAG on 10-20% gradient tricine PAAG (Novex). Gels were dried and exposed to film with an intensifying screen at –70°C.

IκBα cDNA sequencing

IκBα cDNA was obtained by RT-PCR from total RNA using previously described primers and conditions (Emmerich et al., 1999) except the modification in sense primer in the fourth pare of primers. We used the primer: 5′-GCTCAGGAGCCCTGTAATGGCC-GGACTG-3′. PCR products were resolved on 1.5% agarose gel, extracted by QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) and subjected to direct sequencing.

Transfection of cell lines and luciferase activity

Prostate cells were plated on 35 mm dishes and at 50% confluence were co-transfected by Tfx-50 reagent (Promega Corp.) with the following constructs: κB-luciferase reporter – *Fireflight* luciferase (FL) under promoter with three copies of conventional κB site (Clontech Laboratories Inc., Palo Alto, CA); pRL-null construct – *Renilla* luciferase (RL) under minimal promoter (Promega); MMTV.luciferase reporter – *Fireflight* luciferase (FL) under control of MMTV promoter (Clontech); kinase-inactive mutants of either IKKα (IKKαK44M) or IKKβ (IKKβK44M) which work in dominant-negative (d.n.) fashion; and IκBα d.n. mutant. Plasmids with IKK mutants were described earlier (Mercurio et al., 1997) and kindly provided by F. Mercurio (Signal Pharmaceutical Inc., San Diego, CA). Plasmid with the IκBα d.n. mutant lacking serine 32 and serine 36 (Van Antwerp et al., 1996) was a kind gift of I. Verma (Salk Institute, San Diego, CA). Tfx-50 reagent (2.25 μl/μg of plasmid DNA) and the plasmid DNAs (all at a dose of 2 μg/dish) κB.Luc, pRL-null, IKKα d.n., IKKβ d.n., and IκBα d.n. were added to the dishes in antibiotic-free, serum-free medium. 24 hours after transfection, prostate cells were harvested in the lysis buffer and the luciferase activity was measured by dual luciferase assay (Promega) as recommended by the manufacturer. FL activity was normalized against RL activity to equalize for transfection efficacy.

Northern blot analysis

Total RNA from freshly harvested cells was isolated by TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and subjected to northern blotting. 20 μg of total RNA was resolved in a 1% agarose/6% formaldehyde gel. The RNA was transferred to nylon membranes and probed for *IκBα* and *IL-6*. The membranes were also hybridized with a 7S RNA probe to verify that equal amounts of RNA were loaded and transferred. The DNA probes were prepared by random-primed reactions using the complete coding sequence of human *IκBα* and *IL-6* cDNAs (ATCC, Rockville, MD).

P65 immunostaining of prostate tumors

Prostate tissues were obtained from white male patients at the age 40-82 years during biopsy or surgery to remove prostate tumors. Paraffin sections of formalin-fixed prostate carcinoma samples with verified diagnosis and surrounding normal tissues were used for immunostaining. After microwave Ag retrieval and blocking with 5% nonfat milk in PBS, tissues were incubated with primary rabbit polyclonal p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary biotinylated anti-rabbit IgG. Immunostaining was visualized with streptavidin-alkaline phosphatase/histo mark red reagent (Kirkegaard & Perry, Gaithensburg, MD). Sections were counterstained in Mayer's hematoxylin.

Adenovirus infection and apoptosis detection

Prostate cells were plated on 35 mm dishes and at 50% confluence were infected with type 5 recombinant Adenovirus (AdV) construct AdV-d.n.IκBα encoding green fluorescent protein (GFP) and mutant human IkBα protein with substitution of serines 32 and 36 to alanines (32A36A) or adenovirus encoding only GFP (AdV-control). AdV-d.n.IkBα virus with deletions of E1 and E3 was generated using the AdEasy1 system. AdEasy1 system was a generous gift of T.-C. He (The Howard Hughes Medical Institute, Baltimore, MD) (He et al., 1998). Mutations of IkBα were constructed by site-directed mutagenesis with the Bio-Rad Muta-Gene Phagemid In Vitro Mutagenesis system (Bio-Rad Laboratories, Hercules, CA) as described (Whiteside et al., 1995). IkBα mutant has an N-terminal tag (ADRRIPGTAEENLQK) derived from the Equine Infectious Anemia Virus (EIAV) tat protein. Control E1/E3-deleted AdV 5 with GFP (AdV-control) was purchased from Quantum Biotechnologies (Montreal, QC, Canada). Adenoviruses were purified by CsCl gradient centrifugation. Cells were infected with adenoviruses (10^9 vp/dish) in 700 μl of medium with 0.5% serum overnight. 24 hours after infection cells were treated with 7.5 ng/ml TNF-α (R&D Systems, Minneapolis, MN) for 10 hours. Apoptosis was determined morphologically by counting the number of blebbing cells out of 200 fluorescent cells per slide. In addition, we used PARP proteolysis to determine apoptosis. Adherent cells and detached floaters were combined for whole-cell protein extract preparations. PARP cleavage was estimated by western blot analysis with PARP antibody (PharMingen, San Diego, CA).

Data in all figures are shown as results of the representative experiments. All experiments were repeated at least three times.

Results

NF-κB is constitutively activated in PC androgen-independent cell lines In the present study we compared NF-κB function in primary prostate epithelial cells, and androgen-dependent and androgen-independent PC cell lines, which in a way represent sequential stages of prostate tumor development towards hormone-independent growth.

To evaluate NF-κB DNA-binding activity, we performed an electrophoretic mobility shift assay (EMSA) using nuclear protein extracts. We found a strong increase of κB DNA-binding in androgen-independent DU145, PC3 and JCA1 cell lines compared with normal prostate epithelial cells and androgen-dependent LNCaP and MDA PCa 2b cells (Fig. 1A). It is important to note that κB DNA-binding was higher in androgen-independent CL2 cells derived from androgen-dependent LNCaP cells via an in vitro androgen deprivation (Fig. 1A). Significantly, the level of NF-κB binding in androgen-independent cells was similar to one in LNCaP cells treated with TNF-α (Fig. 1A, last lane). To rule out the general effects that some transcriptional regulators in androgen-independent PC cells have in κB-binding, we performed EMSA with Sp1 oligonucleotide. As shown in Fig. 1C, Sp1 binding activity did not correlate with androgen-dependence of growth. It was equally low in androgen-dependent LNCaP cells, their androgen-independent counterpart CL2, and androgen-independent DU145 cells. By contrast, Sp1 binding was much higher in androgen-dependent MDA PCa 2b cells and in androgen-independent JCA1 cells. Thus, NF-κB was specifically upregulated in androgen-independent PC cells.

Analysis of nuclear κB-binding complexes was done using electrophoretic mobility super shift assay (EMSSA). As shown in Fig. 1B, the incubation of nuclear extracts from PC3 cells with anti-p50 antibody removed both complexes while incubation of extracts with anti-p65 antibody removed only the upper complex. Similar results were obtained by EMSSA for other PC cells (data not shown). Incubation of extracts with anti-p52, anti-c-Rel, and anti-RelB antibodies did not affect complex mobility, although those antibodies properly worked in EMSSA with positive control samples (data not shown). In addition, western blot analysis showed the lack of c-Rel and RelB protein expression in normal and malignant prostate cells (data not shown). Thus, in all studied prostate cells the constitutive complexes were represented by p65/p50 and p50/p50 dimers.

To study NF-κB functional activity we performed transient transfection of primary prostate epithelial cells obtained from

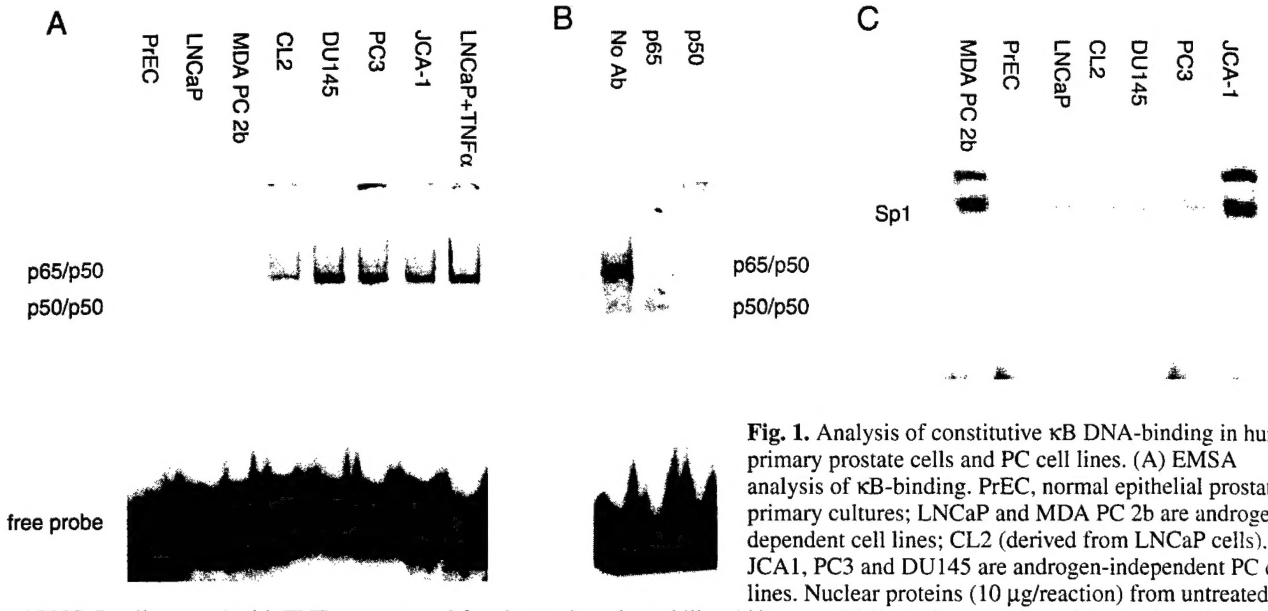

Fig. 1. Analysis of constitutive κB DNA-binding in human primary prostate cells and PC cell lines. (A) EMSA analysis of κB-binding. PrEC, normal epithelial prostate primary cultures; LNCaP and MDA PC 2b are androgen-dependent cell lines; CL2 (derived from LNCaP cells), JCA1, PC3 and DU145 are androgen-independent PC cell lines. Nuclear proteins (10 μg/reaction) from untreated cells and LNCaP cells treated with TNF-α were used for electrophoretic mobility shift assay (EMSA). Composition of dimers is indicated. Data are shown for one representative experiment. (B) Identification of nuclear κB-binding complexes by EMSSA. Nuclear proteins from PC3 cells were incubated with a labeled κB oligonucleotide and antibodies against p50 and p65 proteins. DNA binding activity was analyzed by EMSA. Composition of dimers is indicated. (C) EMSA analysis of Sp1-binding. Nuclear proteins (10 μg/reaction) from the same cells as in Fig. 1A, were used for EMSA with Sp1 oligonucleotide. Composition of dimers is indicated. Data are shown for one representative experiment.

two different donors and several PC cell lines with exogenous κB-responsive gene, κB-luciferase reporter. The results of these experiments presented in Fig. 2A, in general correlated well with the EMSA results: the basal activity of κB reporter was much higher in androgen-independent PC3 and JCA1 cells than in primary prostate cells and LNCaP cells.

We also evaluated the expression of κB-responsive endogenous genes IκBα and IL-6 genes which are tightly regulated by NF-κB in different cells and contain several κB-binding sites in the promoter region (Le Bail et al., 1993; Zhang et al., 1994). As expected, the results of northern blot analysis demonstrated high constitutive levels of IκBα and IL-6 mRNA expression in androgen-independent DU145 and PC3 cells (Fig. 2B). The levels of IκBα and IL-6 mRNA expression in JCA1 cells were comparable with those in androgen-dependent cells possibly due to the absence of some other factors necessary for transcription of these genes in JCA1 cells.

To extend our observation of increased NF-kB activity in PC cells lines, we performed p65 immunostaining of ten samples of human PC obtained during biopsy and two samples of PC with apparently normal surrounding prostate tissues obtained during prostatectomy. The results clearly showed that p65 was overexpressed in the epithelial component of tumors in comparison with the surrounding tissues. Moreover, p65 was localized both in cytoplasm and in the nuclei of cells in PC: 23±8% of nuclei in PC were p65-positive compared to

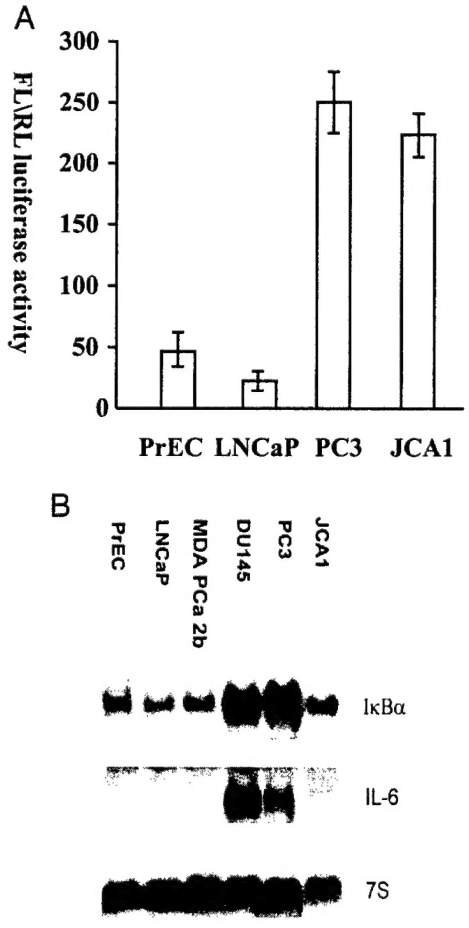

Fig. 2. Analysis of constitutive NF-κB transcriptional activity in human primary prostate cells and PC cell lines. (A) Constitutive activity of κB-luciferase reporter. Prostate cells were cotransfected with κB-luciferase reporter and pRL-null construct. Luciferase activity was measured 24 hours after transfection in untreated prostate cells by dual luciferase assay. Data are shown as FL/RL ratio for one representative experiment. (B) Northern blot analysis of constitutive *IκBα* and *IL-6* genes expression. Northern blots containing total RNA (20 μg/lane) from untreated normal prostate and PC cells were probed for expression of *IκBα* and *IL-6* genes. The membranes were also hybridized with a 7S RNA probe as a control for equal RNA loading.

Table 1. Nuclear expression of p65 in human prostate carcinomas and in apparently normal surrounding prostate tissue

Prostate carcinomas		Normal surrounding prostate	
Sample number	p65 nuclear staining*	Sample number	p65 nuclear staining*
1‡	25%	1	9.5%
2‡	28%	2	10.5%
3	11%		
4	14%		
5	15%		
6	18%		
7	16%		
8	21%		
9	37%		
10	28%		
11	29%		
12	30%		

*The number of p65-positive nuclei is presented as a percentage of 200-300 nuclei evaluated in prostate epithelial cells per section.
‡Samples were obtained during prostatectomy.

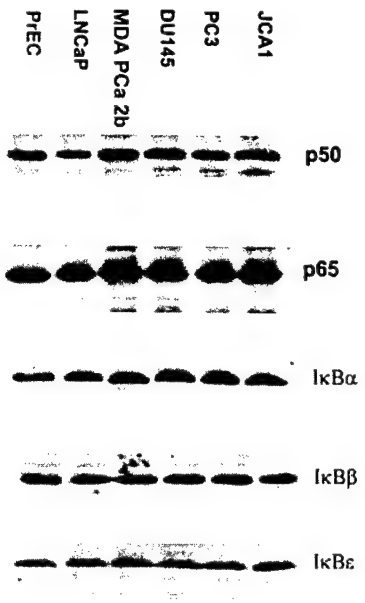

Fig. 3. Western blot analysis of NF-κB and IκB protein expression in human primary prostate cells and PC cell lines. Western blots containing whole cell protein extracts (10 μg/lane) from untreated cells were probed for expression of p50, p65, IκBα, IκBβ and IκBε.

10.5±0.7% of nuclei in normal tissues (Table 1). Translocation of p65 to the nucleus strongly suggests that NF-κB is activated in prostate tumors. Unfortunately hormone-dependence of tumors could not be assessed because we used biopsies and surgically removed PC tissues from untreated patients.

Activation of NF-κB in PC cell lines is not caused by changes in NF-κB and IκB expression or structure

As a first step to elucidate the mechanism(s) leading to the NF-κB activation in androgen-independent PC cells we have analyzed the expression of p50, p65, IκBα, IκBβ and IκBε in comparison with primary prostate cells and androgen-dependent LNCaP and MDA PCa 2b cells. Western blotting of whole-cell protein extracts has not revealed any significant changes in the level of expression of p50, p65 or IκBs in all studied cells (Fig. 3). None of the studied cells expressed RelB or c-Rel (data not shown). The analysis of molecular weights of NF-κB and IκB proteins in PC cells did not reveal any deviations from the expected sizes, suggesting that there were no large alterations of NF-κB and IκB proteins in all studied cell lines.

It was shown that IκBα protein is truncated/mutated in cell lines from some hematopoetic tumors (Rayet and Gelinas, 1999; Emmerich et al., 1999; Cabannes et al., 1999). To address the question whether NF-κB activation in androgen-independent PC cells could be a consequence of mutations or small deletions in the *IκBα* gene, we performed sequencing of IκBα cDNAs obtained by RT-PCR from JCA1, PC3 and DU145 cells. The direct sequencing of IκBα cDNA has not predicted any amino acid substitutions in IκBα protein in those cell lines with constitutive NF-κB activation.

Even though we showed that IκBα is not mutated in PC cells, we could not rule out that other IκB proteins are mutated or functionally impaired in those cells. Thus, in our next set of experiments we addressed the question whether NF-κB activation in androgen-independent PC cell lines is a result of altered interaction between NF-κB and IκB molecules using the universal inhibitor of all IκB degradation, MG132 (Sun and Carpenter, 1998). We expected that MG132, which blocks proteasome-dependent IκB proteolysis, will inhibit basal κB DNA binding if interaction between NF-κB and IκBs in androgen-independent cells is normal. As shown in Fig. 4, MG132 indeed strongly inhibited basal κB DNA binding in PC3 and DU145 cells 30-60 minutes after treatment (Fig. 4). MG132 also decreased κB DNA binding in JCA1 cells 1 hour after treatment (Fig. 4). These results suggest that NF-κB is normally controlled by IκBs in PC cells. Thus, the increased basal NF-κB activity in these cells is not a result of expression of mutated IκB or mutated NF-κB proteins constitutively present in the nucleus.

Increased IκBα phosphorylation and turnover in PC androgen-independent cell lines

Induced NF-κB activation requires IκBα phosphorylation at Ser32 and Ser36 followed by IκBα ubiquitination and degradation (Whiteside et al., 1995; Traenckner et al., 1995). To study IκBα turnover in PC cell lines we used several experimental approaches. First we compared the level of IκBα phosphorylation in different PC cells by western blotting with antibodies directed against IκBα phosphorylated at Ser32.

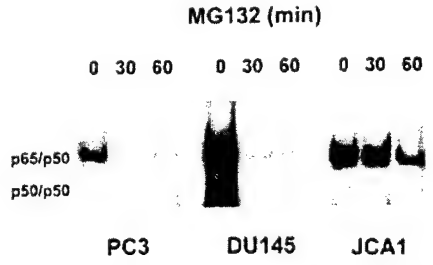

Fig. 4. Proteasomal inhibitor MG132 decreased κB DNA binding in androgen-independent PC cells. Androgen-independent cell lines were treated with proteasomal inhibitor MG132 (7.5 μg/ml) for 30-60 minutes. Nuclear proteins (5 μg/reaction) were used for EMSA.

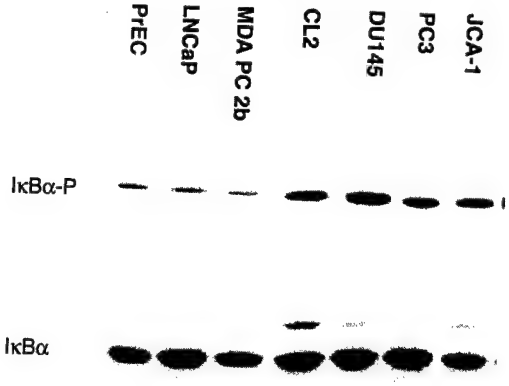

Fig. 5. Analysis of IκBα phosphorylation in androgen-independent PC cells. Western blots containing whole cell protein extracts (10 μg/lane) from untreated cells were probed for expression of IκBα and IκBα-P. Data are shown for the one representative experiment.

Results presented in Fig. 5 clearly show that IκBα is heavily phosphorylated in androgen-independent DU145, PC3, and JCA1. We would like to emphasize that, in several experiments, the highest level of IκBα-P was found in DU145 cells with the highest constitutive κB activity. We also found that level of IκB-α phosphorylation was higher in androgen-independent CL2 cells than in LNCaP cells from which they were derived (Fig. 5).

Further, we assessed the rate of constitutive IκBα phosphorylation in LNCaP cells with low and DU145 cells with high constitutive activity of NF-κB. To evaluate the rate of IκBα phosphorylation we used proteasomal inhibitor MG132 to block degradation of phosphorylated IκBα (Sun and Carpenter, 1998). MG132 treatment resulted in accumulation of phosphorylated IκBα protein in both cell lines, however the rate of IκBα-P accumulation was faster and the final amount of the phosphorylated IκBα protein was much higher in DU145 cells compared with LNCaP cells (Fig. 6).

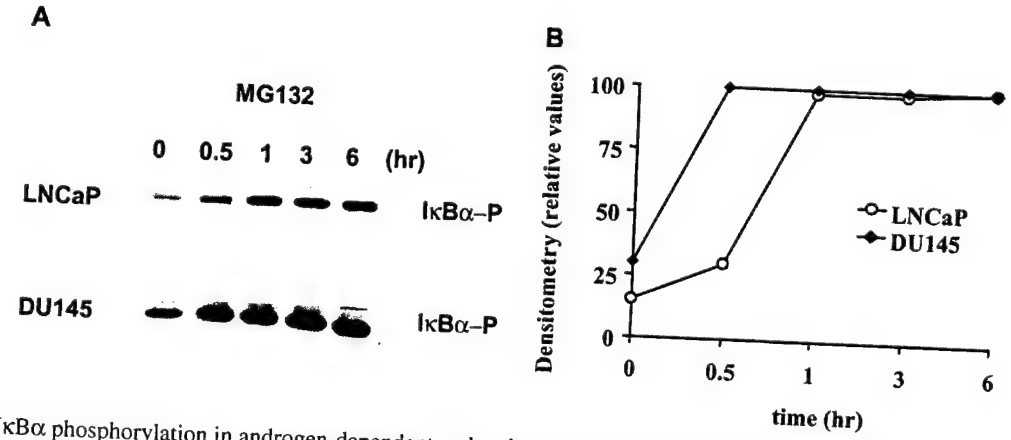

Fig. 6. The rate of IκBα phosphorylation in androgen-dependent and androgen-independent PC cell lines. Indicated cell lines were treated with proteasomal inhibitor MG132 (7.5 μg/ml) for 0.5-6 hours. (A) Western blots containing cytosol proteins (10 μg/lane) were probed for expression of IκBα-P. (B) Western blots shown in A are plotted as a percentage of the maximum IκBα-P expression level. Abscissa: time after MG132 treatment (hours). Ordinate: relative amount of IκBα-P. Data are shown for the one representative experiment.

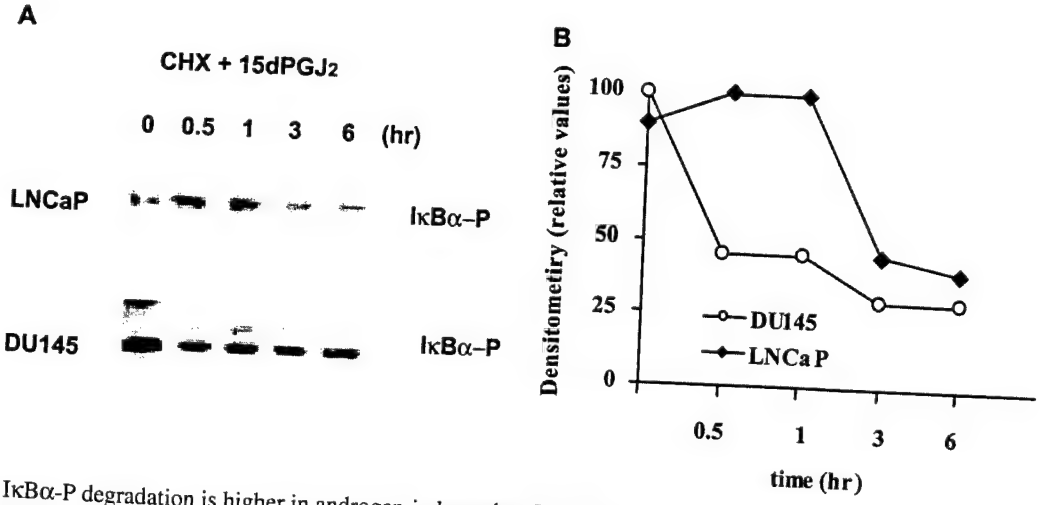

Fig. 7. The rate of IκBα-P degradation is higher in androgen-independent PC cells. Indicated cell lines were treated with cycloheximide (CHX, 10 μg/ml) in combination with 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2) for 0.5-6 hours. (A) Western blots containing cytosol protein extracts (10 μg/lane) were probed for expression of IκBα-P. (B) Western blots shown in A are plotted as a percentage of the initial IκBα-P expression level. Abscissa: time after treatment (hours). Ordinate: relative amount of IκBα-P. Data are shown for the one representative experiment.

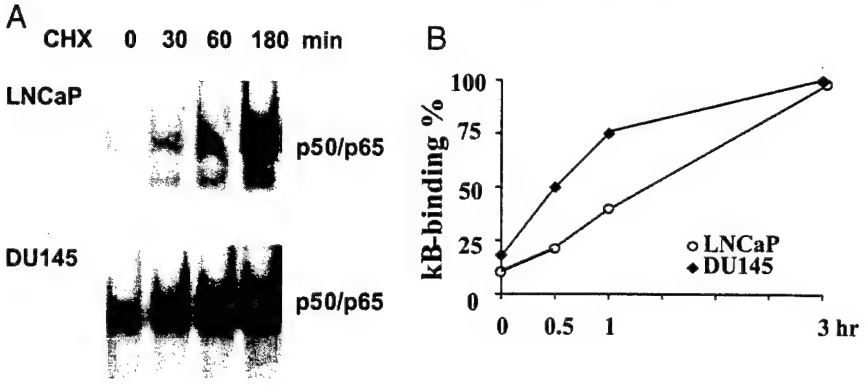

Fig. 8. Effect of cycloheximide on NF-κB binding in PC cells. Indicated cell lines were treated with CHX (10 μg/ml) for 0.5-3 hours. Nuclear proteins (5 μg/reaction) from cells were used for electrophoretic mobility shift assay (EMSA). (A) NF-κB binding activity. (B) NF-κB binding activity shown in A is plotted as a percentage of the maximum (at 3 hours of CHX treatment) p65/p50 binding level. Abscissa: time after treatment (minutes). Ordinate: relative amount of NF-κB binding. Data are shown for the one representative experiment.

Next we evaluated the rate of IκBα-P degradation in the same PC cell lines using cycloheximide (CHX), an inhibitor of protein synthesis, combined with cyclopentenone prostaglandin J2 (15dPGJ2), an inhibitor of IKK (Rossi et al., 2000). Under these conditions, de novo synthesis of IκBα as well as phosphorylation of pre-existing IκBα were blocked. Western blot analysis of pre-existing IκBα-P degradation demonstrated that the rate of degradation of IκBα-P was significantly higher in DU145 cells than in LNCaP cells (Fig. 7).

Facilitation of degradation of IκBα due to the blockage of its de novo synthesis, was expected to result in the translocation of NF-κB into the nucleus. Indeed, CHX treatment increased κB binding both in DU145 and LNCaP cells. The comparison of time curves for NF-κB activation by CHX confirmed that IκB degradation occurs at a considerably higher rate in DU145 cells than in LNCaP cells (Fig. 8).

We also directly assessed the time of IκBα half-life in those two PC cell lines using pulse-chase analysis of metabolically labeled IκBα (Fig. 9). We found that IκBα was more than twice as stable in LNCaP cells (IκBα half-life was about 60 minutes) as in DU145 cells. Conclusively, the comparative analysis of IκBα-P phosphorylation and degradation indicates that IκBα turnover is significantly greater in androgen-independent PC cells; this suggests that IKK activity should be higher in those cells.

Instability of IκBα correlates with constitutive IKK activity in PC androgen-independent cell lines

Recently several IκB kinases (IKK) that phosphorylate IκB proteins in response to diverse NF-κB activators have been identified. The IKKα and IKKβ are the major inducible IKKs (Maniatis, 1997). Western blot analysis of whole cell protein extracts from primary prostate cells and five PC cell lines with antibodies against IKKα/IKKβ did not show significant alterations in the expression of those proteins (Fig. 10A). To determine the activity of endogenous IKKs in prostate cells we performed an in vitro kinase assay. As a positive control we used a protein extract from LNCaP cells stimulated by TNF-α. The data presented in Fig. 10B demonstrate that as predicted, the constitutive IKK activity was higher in the three androgen-independent cell lines, than in primary prostate cells and androgen-dependent cell lines. Thus, constitutive IKK activation appears to be responsible for high rate of IκBα phosphorylation and ultimately for NF-κB activation in androgen-independent PC cells.

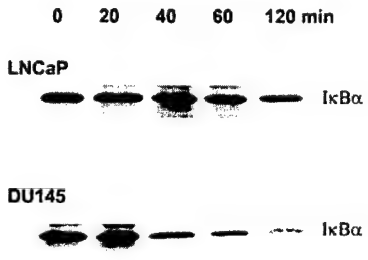

Fig. 9. Pulse-chase analysis of IκBα degradation in PC cell lines. LNCaP and DU145 cells were metabolically labeled with ^{35}S-Met-Cys and harvested at indicated time points. IκBα was immunoprecipitated, resolved on 12.5% denaturating PAAG and transferred to membrane. Dried membrane was subjected to radiography.

Effect of IKK d.n. constructs on the basal level of NF-κB activity in prostate cells

To further explore the role of IKKs in constitutive NF-κB

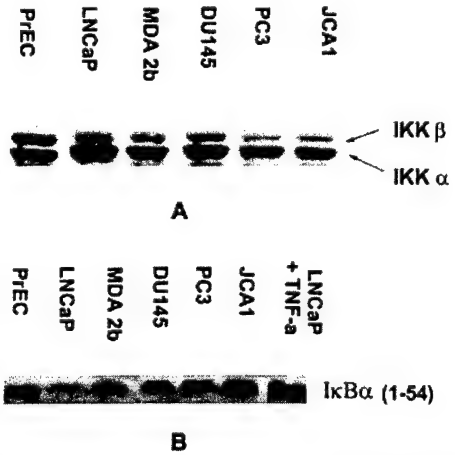

Fig. 10. Analysis of IKK expression and IKK activity in normal prostate cells and PC cell lines. (A) Western blot analysis of IKKα and IKKβ expression. Western blots containing whole cell protein extracts from untreated cells (10 μg/lane) were probed for expression of IKKα and IKKβ. (B) Analysis of IKK activity. Protein extracts from untreated cells were immunoprecipitated with a combination of IKKα and IKKβ antisera, and used for in vitro kinase reaction. Protein extract from LNCaP cells stimulated by TNF-α (7.5 ng/ml, 10 minutes) was used as a positive control.

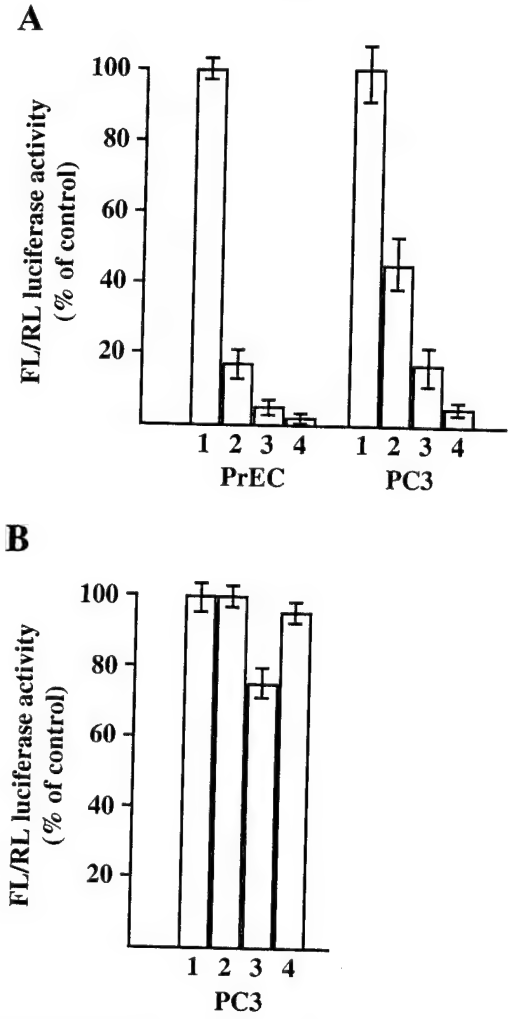

Fig. 11. Effect of d.n. IKK mutants on NF-κB constitutive activity in normal prostate cells and the PC3 cell line. (A) Prostate cells were co-transfected with κB-luciferase, pRL-null and (1) control vector; (2) IKKα d.n. mutant; (3) IKKβ d.n. mutant; and (4) IκBα d.n. mutant. (B) Prostate cells were co-transfected with MMTV-luciferase, pRL-null and (1) control vector; (2) IKKα d.n. mutant; (3) IKKβ d.n. mutant; and (4) IκBα d.n. mutant. Luciferase activity was measured 24 hours after transfection in untreated prostate cells by dual luciferase assay. Data are shown as FL/RL luciferase activities ratio (% to control) for one representative experiment. PrEC, normal epithelial prostate primary cultures.

activation in malignant prostate cells we studied the effect of kinase-inactive mutants of either IKKα (IKKαK44M) or IKKβ (IKKβK44M) on the constitutive NF-κB transcription activity in normal and malignant PC cells in comparison with the effect of IκBα d.n. mutant. Those IKK mutants are not able to phosphorylate IκBs and were shown to block IKK activity in a dominant-negative fashion in such cells as HeLa and 293 human embryonic kidney cells (O'Mahony et al., 2000). As shown in Fig. 11A, both mutants inhibited constitutive luciferase activity in normal and malignant PC3 prostate cells in a similar way, with IKKβ mutant being a more potent inhibitor for constitutively active NF-κB. The effect of the IKKβ mutant was comparable with the effect of IκBα d.n. mutant. The inhibitory effect of IKK mutants on the κB.Luc reporter was specific: IKKβ and IKKα mutants did not affect significantly the constitutive activity of MMTV.Luc in PC3 cells (Fig. 11B).

Effect of IκBα d.n. construct on basal and induced apoptosis in prostate cells

We have extended our study further and studied the biological consequences of NF-κB blockage in PC cells with the high and low constitutive NF-κB activity. We have chosen for these experiments IκBα mutant, which was able to block significantly (up to 90-95%) NF-κB activity in the luciferase reporter assay (Fig. 11). Apoptosis was determined morphologically and by poly(ADP-ribose) (PARP) cleavage. Caspase-mediated cleavage of PARP inactivates this enzyme and inhibits its ability to respond to DNA strand breaks for repair. PARP cleavage is now recognized as one of the most sensitive markers of caspase-mediated apoptosis. We found that NF-κB blockage in LNCaP cells by the IκBα d.n. mutant resulted in massive apoptosis comparable with the apoptosis induced by TNF-α. We observed profound cell retraction, rounding and detachment 24-48 hours after infection. PARP cleavage was similarly increased in LNCaP cells infected with AdV-d.n. IκBα and in LNCaP cells treated with TNF-α (Fig. 12A, lanes 2,5). Moreover, those treatments resulted in reduced expression of full-length PARP, and consequently the ratio of cleaved PARP/total PARP was dramatically affected in cells with blocked NF-κB, and especially in cells with blocked NF-κB treated with TNF-α. It is interesting that NF-κB blockage in PC3 cells resulted in significant apoptosis only when it was combined with TNF-α treatment (Fig. 12A,B). Indeed, the ratio of cleaved PARP/total PARP was high only in PC3 cells with blocked NF-κB treated with TNF-α (Fig. 12A, lane 3). Consistently, 30-40% of TNF-α-treated PC3 cells with blocked NF-κB cells demonstrated characteristic blebbing (Fig. 12B, 3). Neither infection with AdV-d.n. IκBα alone nor treatment with TNF-α alone induced changes in morphology of PC3 cells (Fig. 12B, 1,2).

Discussion

This is the first study to develop a comprehensive and detailed picture of changes in basal NF-κB activity in a panel of prostate cells including primary prostate epithelial cells, two androgen-dependent and four androgen-independent PC cell lines. We found that NF-κB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, JCA1 as well as androgen-independent CL2 cells derived from LNCaP androgen-dependent cells. Thus, we confirmed the recent finding of a high NF-κB activity in some PC cell lines (Palayoor et al., 1999). Our results are also in agreement with recent findings on persistent activity of NF-κB in several other human tumors and tumor cell lines (Rayet and Gelinas, 1999; Baldwin, 1996; Wang et al., 1999; Bours et al., 1994; Nakshatri et al., 1997; Sovak et al., 1997; Visconti et al., 1997; Dejardin et al., 1995).

It is important to mention that amplification, overexpression and rearrangements of most genes coding for Rel/NF-κB factors have been found in hematopoietic tumors and could underlie the constitutive NF-κB activation (Rayet and Gelinas, 1999). However, the most frequent finding in solid tumors and

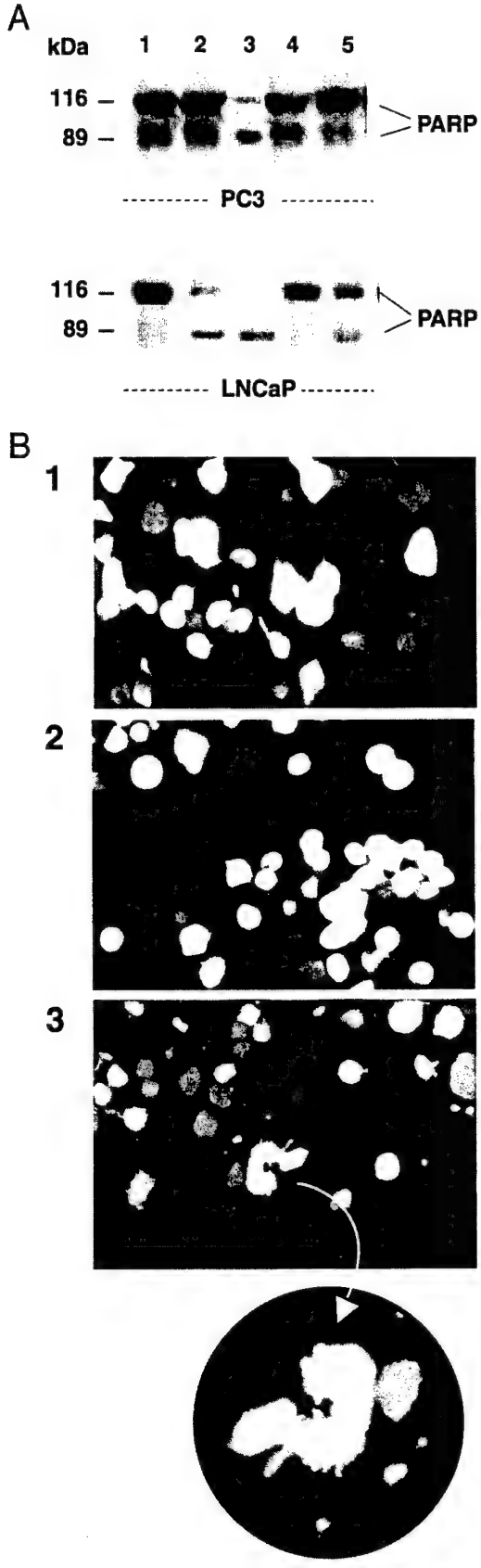

Fig. 12. Effect of IκBα d.n. mutant on apoptosis in prostate cells. (A) Western blot detection of PARP cleavage. PC3 and LNCaP prostate cells were infected with adenovirus expressing GFP and IκBα mutant lacking Ser32 and Ser36 (AdV-d.n.IκBα) or adenovirus expressing only GFP (AdV-control). 24 hours later cell cultures were left untreated or treated with TNF-α (7.5 ng) for 10 hours. PARP cleavage was detected by western blotting with antibody that detects the full length PARP (116 kDa) and PARP cleavage product (85 kDa). Adherent cells and detached floaters were combined for whole-cell lysate preparations. (1) Untreated cells; (2) AdV-d.n.IκBα-infected cells; (3) AdV-d.n.IκBα-infected cells treated with TNF-α; (4) AdV-control-infected cells; (5) AdV-control-infected cells treated with TNF-α. (B) Effect of IκBα d.n mutant on morphology of PC3 cells. Micrographs (×300) depicting representative morphological response of PC3 cells 48 hours after infection: (1) with AdV-control; (2) with AdV-d.n.IκBα; and (3) with AdV-d.n.IκBα and treated with TNF-α. Note numerous blebbing cells in cell cultures treated with TNF-α.

cell lines derived from solid tumors was the overexpression of p50 and p52 proteins (Rayet and Gelinas, 1999; Dejardin et al., 1995). p50 and p52 proteins have low transactivation activity, thus the biological role of p50 and p52 homodimers appears to be ambiguous (Budunova et al., 1999). The participation of RelA in solid tumors is the subject of many debates. RelA exhibits strong transactivation potential, however, alteration of RelA expression/function in solid tumors or cell lines derived from solid tumors has been only rarely reported (Rayet and Gelinas, 1999). Significantly, we found that the activation of p65/RelA-containing NF-κB complexes with the highest transactivation potential among other NF-κB dimers, was specific for PC cell lines and occurred without p65 or p50 overexpression in androgen-independent PC cells. In this regard it is important that nuclear p65 expression was increased in prostate carcinomas compared to surrounding apparently normal tissues.

The altered expression of IκBs as well as mutations in IκB genes in tumor cells are implicated in the constitutive activation of NF-κB (Rayet and Gelinas, 1999; Emmerich et al., 1999; Cabannes et al., 1999). However, the results of our experiments strongly suggest that constitutive activation of NF-κB in PC cells is not a consequence of either altered expression or large rearrangements or mutations in NF-κB/IκB genes. Indeed, we did not find any changes in the level of expression of p65, p50 and three major IκB proteins (IκBα, IκBβ and IκBε) as well as deviations from expected sizes of those molecules in PC cells with activated NF-κB. Further, direct sequencing of IκBα cDNA has not predicted any mutations of the IκBα protein in cell lines with constitutive NF-κB activation. We cannot presently rule out the presence of mutations in IκBβ, IκBε, p50 or RelA genes in DU145, PC3 and JCA1 cells. However, our experiments with different NF-κB inhibitors and activators provided indirect evidence that NF-κB is normally controlled by IκBs and fully functional in those PC cells. Indeed, the constitutive activity of NF-κB in DU145, PC-3 and JCA1 cells was inhibited by the IKKα d.n. mutant, IKKβ d.n. mutant and by a proteasomal inhibitor MG132 that effectively blocks degradation of all IκB proteins (Sun and Carpenter, 1998). The analysis of the sensitivity of PC cells to the standard NF-κB inducers such as TNF-α, LPS and TPA, revealed that, in contrast to the Hodgkin lymphoma cells (Krappmann et al., 1999), and in spite of the high basal level of NF-κB activity, PC cells are highly sensitive to NF-κB activation (Gasparian et al., 2000).

Another recently described mechanism of NF-κB activation in tumor cells implicates increased IκBα phosphorylation and turnover (Devalaraja et al., 1999; Krappmann et al., 1999). We found that in all studied androgen-independent PC cells, including CL2 cells derived from LNCaP cells, IκBα was heavily phosphorylated. Moreover, IκBα displayed a faster turnover in androgen-independent PC cells than in androgen-dependent PC cells. In addition, by in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent cell lines. It is currently understood that the mechanisms of basal and induced NF-κB activation could be different. Activation of NF-κB through phosphorylation, ubiquitination and proteasome-dependent degradation of IκBs is specific for cells treated with NF-κB inducers (Whiteside and Israel, 1997; Heissmeyer et al., 1999). The mechanisms responsible for the maintenance of the basal NF-κB activity are less clear and may not require IκBα phosphorylation at Ser32/36, ubiquitination or even proteasome-dependent degradation (Miyamoto et al., 1998; Krappmann et al., 1996). Our data strongly suggest that in androgen-independent PC cells, basal NF-κB activation employs a mechanism similar to that for NF-κB activation by inducers such as cytokines. It appears that constitutive NF-κB activity depends on the constitutive aberrant activation of IKKs and consequently, a faster IκBα turnover.

In this regard, it is important to mention that the androgen-independent PC cells produce numerous growth factors and cytokines, that are strong activators of IKK complex and consequently NF-κB. Those cytokines and growth factors include TNF-α, different interleukins, FGF, EGF, NGF, HGF, PDGF and VEGF (Baldwin, 1996; Sun and Carpenter, 1998; Byrd et al., 1999; Gentry et al., 2000; Romashkova and Makarov, 1999). Knowing that the expression of genes encoding certain cytokines, for example *IL-6*, is regulated by NF-κB (Zhang et al., 1994), one could assume that activation of IKK in PC cells involves an established positive autocrine/paracrine loop.

Androgen-independent cell lines used in this study do not express androgen receptor (AR) (Tso et al., 2000; Mitchell et al., 2000). This allows to find an interesting parallel between NF-κB activation in androgen-independent PC cells and estrogen receptor (ER)-deficient breast carcinoma cell lines (Nakshatri et al., 1997; Biswas et al., 2000) and to raise the question of the possible role of NF-κB in the development of growth autonomy and resistance to apoptosis in hormone-independent prostate and breast tumors. It is known that NF-κB is a key anti-apoptotic factor in most cells (Barkett and Gilmore, 1999). It has become clear recently that NF-κB could also play the pro-proliferative role in some cells through direct activation of genes involved in the cell cycle (Biswas et al., 2000; Hinz et al., 1999; Guttridge et al., 1999).

We found that NF-κB blockage resulted in the increased apoptosis in LNCaP cells, and increased sensitivity to apoptosis induced by TNF-α in PC3 cells with high constitutive NF-κB activity. The latter result is in line with the previous finding on the essential role of NF-κB in resistance of PC cells to TNF-α (Sumitomo et al., 1999). The high resistance of PC3 cells with elevated constitutive level of NF-κB, to NF-κB blockage could be explained by the residual NF-κB activity in those cells (data not shown).

It is important to mention that despite some general similarities in the response of prostate cells to androgens and NF-κB inducers, there is an evidence that NF-κB and AR mutually repress each other transcriptional activity. The repression involves either direct protein-protein interaction between AR and p65 or competition for intracellular transcriptional regulators (Palvimo et al., 1996; Valentine et al., 2000). Moreover, crosstalk between signaling mediated by AR and NF-κB also involves transcriptional repression of the *AR* gene by NF-κB (Supakar et al., 1995). This suggests that NF-κB blockage may result in restoration of AR function in PC cells.

In conclusion, the results presented here demonstrate that aberrant IKK activation leads to the constitutive activation of the NF-κB 'survival signaling' pathway in androgen-independent PC cells. Since NF-κB protects prostate cells from apoptosis, possibly stimulates proliferation of PC cells, and plays an important role in the selection for hormone-independence, NF-κB and IKK inhibition may prove useful both in the prevention of PC and in adjuvant therapy. Further studies are needed to identify the affected upstream signaling that results in IKK activation.

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Appendix 2

INTERNATIONAL SYMPOSIUM

NF-κB: Regulation, Gene Expression & Disease

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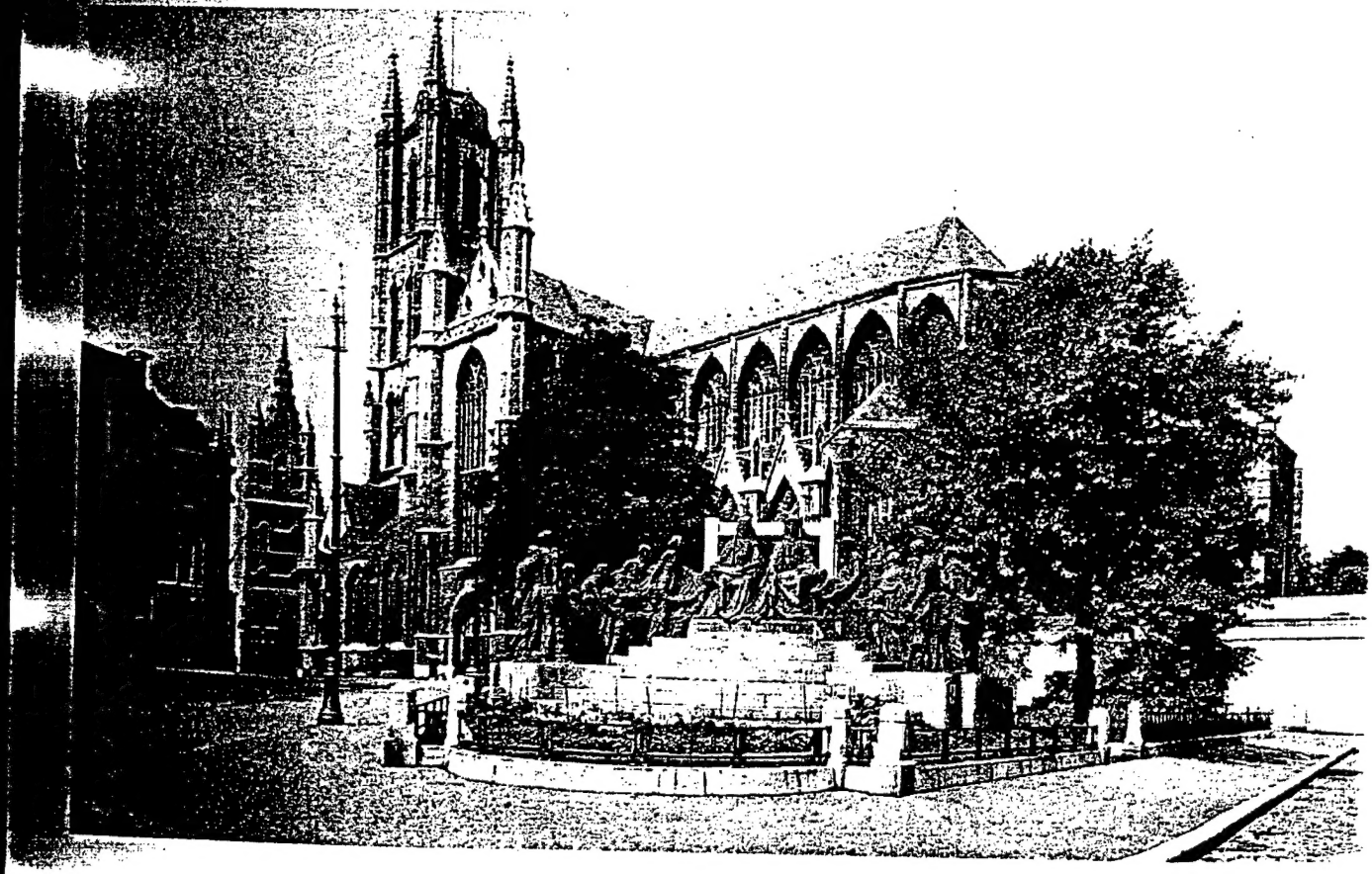

ABSTRACT BOOK

Poster 16

Mechanisms of constitutive NF-kB activation in prostate carcinoma cells

Alexander V. Gasparian, Ya Juan Yao, Dariusz Kowalczyk, Thomas J. Slaga and Irina V. Budunova

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Rel/NF-kB transcription factors are implicated in the control of cell proliferation, apoptosis and transformation. The key to NF-kB regulation is the inhibitory IkB proteins. During response to diverse stimuli, IkBs are rapidly phosphorylated by IkB kinases (IKKs), ubiquitinated and undergo degradation. We have investigated the expression and function of NF-kB, IkB inhibitors and IKKs in normal prostate epithelial cells and prostate carcinoma (PC) cell lines LNCaP, MDA PCa 2b, DU145, PC3, and JCA1. We found that NF-kB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, and JCA1. In spite of strong difference in constitutive kB binding, Western blot analysis did not reveal any significant variance in the expression of p50, p65, IkBs, IKKa, and IKKb between primary prostate cells, androgen-dependent and androgen-independent PC cells. However, we found that in all androgen-independent PC cells IkBa was heavily phosphorylated and displayed a faster turnover than in androgen-dependent LNCaP and MDA PCa 2b cells. By an in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent DU145, PC3 and JCA1 PC cell lines. We have found that PC3 cells produce factor(s) that may be involved in autocrine/paracrine regulation of constitutive NF-kB activity via increasing of IkBa phosphorylation. Our data suggest that increased IKK activation leads to the constitutive activation of NF-kB "survival signaling" pathway in androgen-independent PC cells. This may be important for the support of their androgen independent status and growth advantage.

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Appendix 3

NF-κB: Bench to Bedside

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125 p65-NFκB synergizes with Notch to activate transcription by triggering cytoplasmic translocation of the Nuclear Receptor Corepressor N-CoR.

Espinosa L, Santos S, Inglés-Esteve J, Muñoz-Cánoves P, Bigas A. Molecular Oncology. Institut de Recerca Oncologica. Hospitalet, Barcelona 08907, Spain.

Notch/RBPJκ and Nuclear Factor-κB (NFκB) complexes are key mediators in the progression of many cellular events by activating transcription of specific target genes. Independent observations have shown that activation of Notch dependent transcription generally correlates with inhibition of differentiation. In contrast, activated NFκB complexes are required for progression of differentiation in several systems. Although some interactions between both pathways have been observed, the physiological significance of their connection is unclear. We have now demonstrated that ectopic expression of p65-NFκB protein enhances up to three fold Notch-mediated activation of the Hes1 promoter.This effect does not require NF-κB transcriptional activity and it is independent of the previously described interaction between Notch and p50-NFκB. Furthermore, p65-NF-κB can trigger cytoplasmic translocation of the transcriptional corepressor N-CoR, thus abrogating N-CoR mediated repression of the Hes1 promoter. Thus, we conclude that p65-NFκB can regulate gene expression by a new mechanism that involves functional inhibition of N-CoR.

126 Transgenic NF-kB Reporter Mice Reveal Constitutive NF-kB Activity That Is Required For Central Neuron Survival.

Asha L Bhakar, Laura-Lee Tannis, Christine Zeindler, Maria Pia Russo, Christian Jobin, Sandra MacPherson, Philip A Barker.
Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada. H3A 2B4

The function of NF-kB within the developing and mature central nervous system is controversial. To assess NF-kB activity in neurons *in vivo*, an NF-kB responsive ?-galactosidase minigene was used to generate transgenic mice. Constitutive NF-kB activity was present in transgenic immune organs and TNFα treatment of primary transgenic fibroblasts resulted in inducible ?-galactosidase activity that was inhibited by IkBαM overexpression. Developmental analysis revealed prominent NF-kB activity in the central nervous system that persisted to adulthood, especially within the neo-cortex, olfactory bulbs, amygdala, and hippocampus. In primary dissociated culture of transgenic E16 cortical cells, NF-kB activity was elevated by adenovirus-mediated overexpression of p65/RelA and reduced by adenovirus-mediated overexpression of the IkBαM super-repressor or dominant-negative NIK. Inhibition of NF-kB activity induced cortical neuron death whereas p65/RelA overexpression increased levels of Bcl-xL, IAP1 and IAP2 and conferred potent neuroprotection. Together, these data demonstrate an important role for NF-kB in the survival of CNS neurons.

127 Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells.

Gasparian A.V., Yao Y.J., Lü J., Slaga T.J. and Budunova I.V.
AMC Cancer Research Center, Denver, CO, USA, 80214

Selenium (Se) compounds are potential chemopreventive agents for prostate cancer. There are several proposed mechanisms of their anticancer effect, including enhanced apoptosis of transformed cells. Because the transcription factor NF-κB is often constitutively activated in tumors and is a key anti-apoptotic factor in mammalian cells, we tested whether Se inhibited NF-κB activity in prostate cancer cells. Using sodium selenite and a novel synthetic compound methylseleninic acid (MSeA) that served as a precursor of the putative active monomethyl metabolite methylselenol, we found that both Se forms inhibited NF-κB DNA binding induced by TNF-α and LPS in DU145 and JCA1 cells. The kinetics of NF-κB inhibition was different with a quick and transient inhibition of κB binding by MSeA and a slower but more sustained inhibition by selenite. Both compounds also inhibited κB.Luciferase reporter activity in prostate cells. A key to NF-κB regulation is the inhibitory κB (IκB) proteins that in response to diverse stimuli are rapidly phosphorylated by IκB kinase (IKK) complex, ubiquitinated and undergo degradation, releasing NF-κB factor. We showed that selenite and MSeA inhibited IKK activation, IκB-α phosphorylation and degradation induced by TNF-α and LPS in prostate cells. The extent and persistence of NF-κB inhibition appeared to correlate with the Se effect on growth and survival of prostate cells. These results suggest that Se may target the NF-kB activation pathway to exert, at least in part, its cancer chemopreventive effect in prostate.

This work supported by funding from the DOD Prostate Cancer Research Program DAMD17-01-1-0015.

128 Mechanism of melphalan-induced up-regulation of B7-1 gene expression

Manjula Donepudi, Pradip Raychaudhuri, Margalit B. Mokyr, Department of Biochemistry and Molecular Biology, University of Illinois at Chicago, Chicago, IL, 60612.

We have previously shown that administration of a low dose of the widely-used anticancer drug melphalan (L-phenylalanine mustard, L-PAM) to MOPC-315 tumor bearing mice leads to the acquisition of $CD8^+$ T-cell-dependent tumor-eradicating immunity via a B7-dependent mechanism. In addition, we have shown that the chemotherapy is associated with rapid up-regulation of the surface expression of the costimulatory molecule B7-1 on both tumor cells and host antigen presenting cells. Here we show that *in vitro* exposure of tumor cells or host cells to L-PAM also leads to rapid up-regulation of B7-1 surface expression, and the induced B7-1 molecule is functional. In addition, we show that L-PAM-induced up-regulation of B7-1 surface expression requires *de novo* RNA synthesis and is associated with accumulation of B7-1 mRNA, indicating that the regulation is at the transcriptional level. The effect of L-PAM on B7-1 surface expression can be mimicked by exposing cells to oxidative stress, but not heat shock. Moreover, the antioxidant N-acetyl-L-cysteine (NAC) prevents the L-PAM-induced accumulation of B7-1 mRNA, suggesting that reactive oxygen species are involved in the transcriptional regulation of L-PAM-induced B7-1 expression. Although AP-1 and NF-κB are regarded as redox-sensitive transcription factors, and the promoter/enhancer region of the B7-1 gene contains an AP-1 and NF-κB binding site, exposure of cells to L-PAM leads to rapid and transient activation only of NF-κB, but not AP-1. Moreover, exposure of cells to a cell-permeable peptide that selectively inhibits NF-κB activation by blocking the activation of the IκB kinase complex inhibits the L-PAM-induced B7-1 mRNA accumulation, indicating that NF-κB activation is essential for the L-PAM-induced B7-1 gene expression. Taken together, these results indicate that L-PAM leads to the induction of B7-1 gene expression by activating NF-κB via a pathway that involves reactive oxygen species. These results have important implications for an additional immune potentiating mechanism of anticancer drugs in a clinical setting.